PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 31/725 // (A61K 31/725, 31:715, 31:00)

A1

(11) International Publication Number: **WO 97/20564**

(43) International Publication Date: 12 June 1997 (12.06.97)

(21) International Application Number: PCT/CA96/00793

(22) International Filing Date: 29 November 1996 (29.11.96)

(30) Priority Data:

2,164,260	1 December 1995 (01.12.95)	CA
2,173,037	29 March 1996 (29.03.96)	CA

(71) Applicant *(for all designated States except US)*: HYAL PHARMACEUTICAL CORPORATION [CA/CA]; 2425 Skymark Avenue, Mississauga, Ontario L4W 4Y6 (CA).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: GUSTAFSON, Stefan [SE/SE]; Stackmastarvagen 15, S-756 47 Uppsala (SE).

(74) Agent: HUGHES, Etigson; Suite 200, 175 Commerce Valley Drive West, Thornhill, Ontario L3T 7P6 (CA).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: TARGETING OF DOSAGES OF MEDICINE AND THERAPEUTIC AGENTS AND OTHER GLYCOSAMINOGLYCANS (GAGS)

(57) Abstract

A method of treating a disease or condition in a human treatable by a medicine and/or therapeutic agent which may be transported by an agent to the site in need of treatment in the body and which agent may also transport the medicine and/or therapeutic agent to the liver (by for example, the transport agent binding to receptors on the liver) is provided comprising: (a) administering an effective non-toxic amount of a first agent which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver thereby "down regulating" the liver; and, (b) thereafter administering an effective non-toxic amount of a medicine and/or therapeutic agent and an effective amount of a second agent which is a transport agent and is a different agent from the first agent which binds to the site in need of treatment and would be capable of binding to the sites of the liver if the liver had not been "down regulated" so that its binding capacity for the second agent has been substantially reduced by the up-take by the liver of the first agent administered under sub-paragraph (a) by for example, binding with the scavenger receptors of the liver.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

TARGETING OF DOSAGES OF MEDICINE AND THERAPEUTIC AGENTS AND OTHER GLYCOSAMINOGLYCANS (GAGS)

FIELD OF THE INVENTION

This invention relates to the targeting of medicines and therapeutic agents to sites in the body of a mammal in need of treatment and, in one application, finds use in the treatment of malignant tumours in humans.

BACKGROUND OF THE INVENTION

U.S. Application 07/675,908 owned by Hyal Pharmaceutical Corporation and PCT Application PCT/CA90/00306, Publication No. WO91/04058 also owned by Hyal Pharmaceutical Corporation teaches the use of dosages of at least 10 mg. of forms of hyaluronic acid to transport effective amounts of medicines and/or therapeutic agents to sites in need of treatment in the human body, to penetrate the tissue at the sites in need of treatment, including scar tissue, through all membranes into the cells to be treated.

At page 25, line 17, the PCT Application teaches the additional benefit of using at least about 200 mg. of forms of hyaluronic acid (for example, sodium hyaluronate) in a dosage together with the medicine and/or therapeutic agent for reducing the side effects of the medicine and/or therapeutic agent when administered (such as gastro-intestinal distress, neurological abnormalities, depression, etc. normally associated with the medicine and/or therapeutic agent) even at elevated amounts greater than the usual accepted dosage amounts of the medicine and/or therapeutic agent when administered alone for example, an NSAID (non-steroidal anti-inflammatory agent).

The document continues at page 25, line 26:

> "In addition, the responses that have been observed are superior when the NSAID (for example, Indocid™) is combined with hyaluronic acid demonstrating clearly that the combination is now "targeting" the pathological tissue even when administered by the systemic intravenous route. Thus, it has been observed that patients with neoplastic diseases when receiving in addition to other chemicals (for example, ascorbic acid [Vitamin C], phloretin and anti-cancer drugs), 50 - 200 mg NSAID - hyaluronic acid (sodium hyaluronate) (for example indomethacin and hyaluronic

acid) experience dramatic relief of pain immediately. This is followed within a short period of time by a resolution and resorbtion of neoplastic lesions with an improvement of pulmonary, and liver function if there is tumor present in these organs. Thus, the dead tumor material and the debris and tumor toxins appear to be better eliminated by the body through the action of the macrophages whose activity is enhanced by the addition of the NSAID (or a steroidal anti-inflammatory drug) administered with hyaluronic acid (or salt or other form thereof). Thus Applicants believe that the addition of the NSAID for example with hyaluronic acid (sodium hyaluronate) deblocks the macrophages by preventing enzymatic production of prostaglandin synthetase which blocks macrophage functioning. Thus the hyaluronic acid (and salt and other forms) not only enhance the activity of the NSAID but also reduce any side effects and toxicity that is associated with the use of the prostaglandin synthesis inhibitors."

U.S. Applications Serial No. 08/486,328 and 08/520,591 and PCT Application PCT/CA95/00477, also owned by Hyal Pharmaceutical Corporation, teach the modulation of cellular activity of tissue and cells expressing a high affinity cell-surface receptor for hyaluronic acid by the use of forms of hyaluronic acid. These cell surface receptors comprise adhesion molecule ICAM-1, adhesion molecule CD44 and adhesion molecule HARLEC (Hyaluronic Acid [Hyaluronan] Receptors Liver Endothelial Cells) and regulatory molecule RHAMM (Receptor for HA Mediated Motility), for binding hyaluronan. HARLEC is expressed (produced and put on the cell surface) in liver endothelial cells. The administration of an effective amount of a form of hyaluronic acid to bind with the cell-surface receptors modulates cellular activity of tissues and/or cells expressing such high affinity cell-surface receptors for hyaluronic acid (for example, an adhesion or regulatory molecule) in the human body.

As stated at page 19, line 30 of the Application, the binding capacity of the liver has been found to be so great for hyaluronan that hyaluronan when administered first goes to the liver and if not bound to the liver because the liver has reached its binding capacity for hyaluronan, circulates in the system and collects in for example, a tumour because of

the tumour's receptors' ability to bind with hyaluronic acid (hyaluronan) as a result of the tumour having excess receptors for hyaluronic acid (more than normal tissue and cells).

Therefore, if hyaluronic acid is used as a vehicle for a medicine or therapeutic agent to transport the medicine to a site in the body in need of treatment, unless the combination is administered directly to the site in need of treatment as by injection into a tumour, much of the combination ends up at the liver with lesser amounts at the site in need of treatment, unless and until the liver has reached its binding capacity for hyaluronan.

In an article entitled "Binding of hyaluronate and chondroitin sulphate to liver endothelial cells" by Tovard C. Laurent, et al., Biochem J. (1986) 234, 653-658, the authors discussed the fact that "Circulating sodium hyaluronate (HA) is efficiently taken up and metabolized by the endothelial cells in the liver sinusoids", and that "Chondroitin sulphate (CSA) is also taken up and metabolized by liver endothelial cells". The authors also state:

> "The partial inhibition of HA binding by CSA (Smedared *et al.*, 1984) and the inhibition of CSA binding by HA (Smedared *et al.*, 1985) indicates that the two polysaccharides are recognized by the same receptor. We have now confirmed this hypothesis by the use of oligosaccharides of identical degree of polymerization prepared from the two polymers."

I have now discovered that the liver endothelial cells carry at least two different binding proteins for HA (hyaluronan) including a scavenger receptor that binds to both chondroitin sulphate and hyaluronan, (and other glycosaminoglycan (GAGS)) and, the majority of these different binding proteins (the scavenger receptors on the liver) are inhibited from their take-up of GAGS (Glycosaminoglycans) including chondroitin sulphate (CS) by their being previously bound to, for example, chondroitin sulphate. I have also discovered that the sites in need of treatment for example, metastatic tumours do not carry the same scavenger receptors that bind to chondroitin sulphate. These sites carry receptors that do bind to hyaluronan. Thus, by first administering for example, chondroitin sulphate for take up by the scavenger receptors of the liver, subsequently administered hyaluronan for example, will not be taken by the liver.

It is therefore an object of this invention to provide a novel method of treatment of a disease or condition in a human which method permits

substantially more of the medicine and/or therapeutic agent to be brought to the site in the body in need of treatment.

It is a further object of the invention to target sites in the body in need of treatment so that any medicine or therapeutic agent reaches the site in need of treatment rather than being taken up elsewhere in the body (for example, the liver).

It is still a further object of the invention to provide a novel dosage kit or combination of materials or chemicals which when used one after the other will target the sites in the body in need of treatment for delivery of a medicine and/or therapeutic agent to the site in need of treatment.

It is still a further object of the invention to reduce the amount of the medicine and/or therapeutic agent that would otherwise be normally expected to be used for treating a disease or condition. In this regard, if a transport agent is used to transport the medicine and/or therapeutic agent to the site of the disease and/or condition, it is a further object of the invention to use less of the usual amount of the transport agent (for example, hyaluronan and a pharmaceutically acceptable salt thereof) for transporting the medicine and/or therapeutic agent to transport the medicine and/or therapeutic agent to the site of the treatment. Thus, for example, less cytotoxic medicine (for example, methotrexate, cisplatin and the like) will be needed as an effective dosage amount of the medicine to achieve successful treatment using less than the usual effective dosage amount of the hyaluronan for example, as the transport agent.

Further and other objects of the invention will be realized by those skilled in the art from the following Summary of Invention and Detailed Description of Examples thereof.

SUMMARY OF INVENTION

I have now discovered that while chondroitin sulphate and hyaluronan bind to liver cells and particularly, the scavenger receptors on the liver, chondroitin sulphate does not bind with the receptors on for example, tumours (for example, metastatic tumours) and particularly, the cell surface receptors for hyaluronan comprising, the Regulatory molecule RHAMM (Receptor for HA Mediated Motility), and adhesion molecules ICAM-1, HARLEC and CD44. This has led me to develop my new methods of treatment of disease and conditions including metastatic tumours. By "down regulating" the scavenger receptors on the liver cells by binding them to administered chondroitin sulphate (or other GAGS

[Glycosaminoglycan] such as dextran sulphate, other than a form of hyaluronan) and subsequently administering the form of hyaluronic acid (hyaluronan) with the medicine and/or therapeutic agent, the subsequently administered amounts of hyaluronan (which transport the medicine and/or therapeutic agent) are picked up, not by the liver whose binding capacity has been substantially fulfilled but, by other sites capable of binding with forms of hyaluronan having excess unfilled hyaluronan receptors (such as on metastatic tumours). At the same time, the hyaluronan transports any medicine (or therapeutic agent) to treat the sites in need of treatment (for example, by an effective amount of a cytotoxic agent to treat a tumour). As a result, I have, by the pre-treatment with for example, chondroitin sulphate, found that less of the form of the transport agent for example, hyaluronan that is to be subsequently administered is required than previously used. I have also found that less of the medicine and/or therapeutic agent is required than was used previously to provide a successful treatment of the disease and/or condition.

The amount of the chondroitin sulphate that will "down regulate" the liver cells is preferably in the order of at least about 3-5 mg. of chondroitin sulphate per kilogram of body weight of the patient. However, preferably greater amounts (mg/kg) are administered to "turn off" the liver. Because the liver processes the administered and "taken up" chondroitin sulphate rapidly, less chondroitin sulphate is not as good as more, as after several hours the liver has processed all the chondroitin sulphate. Thus, prolonged "blockage"/"down regulating" or "immobilization" of the liver cells is preferred. Where chondroitin sulphate (equivalent dose 1-2 grams/70 kg person) is administered, the take-up of even small amounts of hyaluronan by the liver (0.5-1 mg/70 kg/person) can be inhibited for an extended time by the administration of the chondroitin sulphate. Thus, the hyaluronan is available to transport the medicine and/or therapeutic agent to the site in need of treatment (for example, methotrexate or cisplatin to a tumour or furosemide to a kidney or other use proposed by the teachings of WO 91/04058 which is incorporated herein by reference.

The amount thereafter required of the transport agent for example, hyaluronan or a pharmaceutically acceptable salt thereof for example, sodium hyaluronate having for example, a molecular weight less than

750,000 daltons may be reduced substantially (for example, to an amount of substantially less than 10 mg/70 kg person such as 0.1 mg/70 kg person) and the amount of medicine and/or therapeutic agent likewise substantially reduced to a mere fraction of what is normally used previously or proposed to be used previously. It may be that with the liver shut down, only micrograms (μg)/kg of the body weight, of the transport agent for example, 20 μg/kg and micrograms (μg) of the medicine for example, depending on the medicine 10 μg/kg of body weight may be only required in the dosage.

Other suitable compounds may be substituted for chondroitin sulphate such as dextran sulphate including other GAGS. Some may be used in substantially the same amounts as with chondroitin sulphate; others may be used in higher or lower amounts. Other GAGS may include Dermatan sulphate, or their Proteoglycan forms, Keratan sulphate. Keratan sulphate and the like, while not technically a glycosaminoglycuronoglycan, will be considered to be included as a GAG herein. Other scavenger receptor ligands may also be used such as acetylated low density lipoproteins (LDL), acids such as poly-inosinic acid and the like.

Subsequent to administering the chondroitin sulphate (for example, after 3 to 4 minutes) (for example, by intravenous administration) the combination of for example, hyaluronan (for example, the amounts, forms and molecular weights taught in U.S. Application 07/675,908 and PCT Application WO91/04058 and Continuation-In-Part Applications Serial No. 08/468,328 filed June 6, 1995 and Serial No. 08/520,591 filed August 30, 1995 (the contents of all four of which are incorporated herein by reference), together with the medicine and/or therapeutic agent (whether excess amounts over and above the normally administered amounts when at least 200 mg/70 kg person of the form of hyaluronic acid is used or the normally used effective amounts of the medicine or therapeutic agents are used or much lesser amounts (μg)/kg) is administered by any suitable means. Because lesser amounts of medicine and/or therapeutic agents and for example, hyaluronan may now be used, the amounts specified above and in the documents which are incorporated herein by reference may be substantially reduced. Because the amount of medicine and/or therapeutic agent is reduced substantially, side effects are substantially reduced. Thus, even the 200 mg.

amount per 70 kg. person of the form of hyaluronic acid will be reduced. In fact, with the liver being "blocked", I have found that 5 μg/250 mg rat (20 μg/1 kg) of hyaluronan targets the site in need of treatment. I have also found that the same order of magnitude (μg/250 gm rat) medicine and/or therapeutic agent in the dosage would be useful for treatment of the site in need of treatment. That is because the liver has now been "blocked". Therefore, the teachings in WO 91/04058 with respect to dosage amounts may now be modified in light of the above for use with this invention.

As stated in Application WO91/04058, whose teachings are incorporated herein by reference:

(i) at page 17, line 3 to page 18, line 16:

"Applicants have now discovered that combinations and formulations (for example an injectable formulation) can be provided for administration to a mammal for the treatment of a disease or condition, which combinations or formulations employ or incorporate as the case may be a therapeutically effective non-toxic amount of a medicinal and/or therapeutic agent to treat the disease or condition (for example a free radical scavenger (for example ascorbic acid (Vitamin C)), Vitamin C (for the treatment of mononucleosis), an anti-cancer agent, chemotherapeutic agent, anti-viral agents for example a nonionic surfactant, e.g. nonoxynol-9 [nonylphenoxy polyethoxy ethanol] found in Delfen™ contraceptive cream, and anionic surfactants (e.g. cetyl pyridinium chloride) and cationic surfactants (e.g. benzalkonium chloride), non-steroidal anti-inflammatory drugs (NSAID) for example indomethacin, naproxen and (+/-) tromethamine salt of ketorolac (sold under the trademark Toradol™) and steroidal anti-inflammatory drugs, anti-fungal agent, detoxifying agents (for example for administration rectally in an enema), analgesic, bronchodilator, anti-bacterial agent, antibiotics, drugs for the treatment of vascular ischemia (for example diabetes and Berger's disease), anti-body monoclonal agent, minoxidil for topical application for hair growth, diuretics (for example furosemide (sold under the trademark Lasix™)),

immunosuppressants (for example cyclosporins), lymphokynes (such as interleukin - 2 and the like), alpha- and-ß-interferon and the like) administered with, or carried in, an amount of hyaluronic acid and/or salts thereof (for example the sodium salt) and/or homologues, analogues, derivatives, complexes, esters, fragments, and/or sub units of hyaluronic acid (preferably hyaluronic acid and salts thereof) sufficient to facilitate the agent's penetration through the tissue (including scar tissue), at the site to be treated through the cell membranes into the individual cells to be treated. When such combinations and formulations are administered to patients suffering from the disease or condition, the disease or condition is unexpectedly improved.

The formulation can be administered among other methods, intravenously, intra arterially, intraperitoneally, intrapleurally, transdermally, on the skin (topically), rectally, orally or by direct injection (for example into a tumor, into an abscess or similar disease focus) or put on a patch to be secured to the skin of the patient. The hyaluronic acid and/or salts thereof and the agent can be administered separately but are administered in sufficient amounts and in an immediate time sequence or interval (preferably concurrently and more preferably simultaneously), preferably at the identical site (e.g. one given intravenously and the other "piggy backed"), to treat the disease or condition."

(ii) at page 25, line 18 to page 26, line 14:

"Thus and according to another aspect of the invention when an NSAID for example indomethacin (dissolved in n-methyl glucamine) or other NSAID is administered with greater than 200mg hyaluronic acid for 1 - 2 mg/kg body weight of the NSAID (in one instance indomethacin and NMG), no major toxic side effects occur such as gastro-intestinal distress, neurological abnormalities, depression, etc., even at elevated amounts of indomethacin (if necessary). If the amount of hyaluronic acid is decreased below that amount, the usual side effects may begin to

reoccur. In addition, the responses that have been observed are superior when the NSAID (for example Indocid™) is combined with hyaluronic acid demonstrating clearly that the combination is now "targeting" to the pathological tissue even when administered by the systemic intravenous route. Thus, it has been observed that patients with neoplastic diseases when receiving in addition to other chemicals (for example ascorbic acid [Vitamin C], phloretin and anti-cancer drugs), 50 - 200 mg NSAID - hyaluronic acid (sodium hyaluronate) (for example indomethacin and hyaluronic acid) experience dramatic relief of pain immediately. This is followed within a short period of time by a resolution and resorbtion of neoplastic lesions with an improvement of pulmonary, and liver function if there is tumor present in these organs. Thus the dead tumor material and the debris and tumor toxins appear to be better eliminated by the body through the action of the macrophages whose activity is enhanced by the addition of the NSAID (or a steroidal anti-inflammatory drug) administered with hyaluronic acid (or salt or other form thereof). Thus Applicants believe that the addition of the NSAID for example with hyaluronic acid (sodium hyaluronate) deblocks the macrophages by preventing enzymatic production of prostaglandin synthetase which blocks macrophage functioning. Thus the hyaluronic acid (and salt and other forms) not only enhance the activity of the NSAID but also reduce any side effects and toxicity that is associated with the use of the prostaglandin synthesis inhibitors.

Examples of agents suitable for use as chemotherapeutic agents are novantrone (Mitoxantrone), Methotrexate, 5-FU (5-Fluouracil), carboplatinum, methyl CCNU administered orally and Mitomycin C."

(iii) at page 26, lines 32 to 37:

"The hyaluronic acid and salts thereof may be utilized at varying doses - 10 to 1000 mg/70 kg person with the optimal doses tending to range between 50 and 350 mg/70 kg individual. As there is no toxicity, the hyaluronic acid can

obviously be administered in a dose excess (for example 3000 mg/70 kg individual) without any adverse effects."

(iv) at page 29, line 27 to page 33, line 31:

"One form of hyaluronic acid and/or salts thereof (for example sodium salt) and homologues, analogues, derivatives, complexes, esters, fragments, and sub units of hyaluronic acid, preferably hyaluronic acid and salts and thereof suitable for use with Applicant's invention is a fraction supplied by Sterivet Laboratories Limited. One such fraction is a 15 ml vial of Sodium hyaluronate 20mg/ml (300mg/vial - Lot 2F3). The sodium hyaluronate fraction is a 2% solution with a mean average molecular weight of about 225,000. The fraction also contains water q.s. which is triple distilled and sterile in accordance with the U.S.P. for injection formulations. The vials of hyaluronic acid and/or salts thereof may be carried in a Type 1 borosilicate glass vial closed by a butyl stopper which does not react with the contents of the vial."

The fraction of hyaluronic acid and/or salts thereof (for example sodium salt) and homologues, analogues, derivatives, complexes, esters, fragments, and sub units of hyaluronic acid, preferably hyaluronic acid and salts thereof may comprise hyaluronic acid and/or salts thereof having the following characteristics:

a purified, substantially pyrogen-free fraction of hyaluronic acid obtained from a natural source having at least one characteristic selected from the group consisting of the following:

i) a molecular weight within the range of 150,000-225,000;

ii) less than about 1.25% sulphated mucopoly-saccharides on a total weight basis;

iii) less than about 0.6% protein on a total weight basis;

iv) less than about 150 ppm iron on a total weight basis;

v) less than about 15 ppm lead on a total weight

basis;

vi) less than 0.0025% glucosamine;

vii) less than 0.025% glucuronic acid;

viii) less than 0.025% N-acetylglucosamine;

ix) less than 0.0025% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.275;

xi) a UV extinction coefficient at 280 nm of less than about 0.25; and

xii) a pH within the range of 7.3-7.9. Preferably the hyaluronic acid is mixed with water and the fraction of hyaluronic acid fraction has a mean average molecular weight within the range of 150,000-225,000. More preferably the fraction of hyaluronic acid comprises at least one characteristic selected from the group consisting of the following characteristics:

i) less than about 1% sulphated mucopolysaccharides on a total weight basis;

ii) less than about 0.4% protein on a total weight basis;

iii) less than about 100 ppm iron on a total weight basis;

iv) less than about 10 ppm lead on a total weight basis;

v) less than 0.00166% glucosamine;

vi) less than 0.0166% glucuronic acid;

vii) less than 0.0166% N-acetylglucosamine;

viii) less than 0.00166% amino acids;

x) a UV extinction coefficient at 257 nm of less than about 0.23;

xi) a UV extinction coefficient at 280 nm of less than 0.19; and

xii) a pH within the range of 7.5-7.7

Other forms of hyaluronic acid and/or its salts, and homologues, derivatives, complexes, esters, fragments and sub units of hyaluronic acid may be chosen from other suppliers, for example those described in the prior art

documents previously referred to. In addition Applicants have successfully employed sodium hyaluronate produced and supplied by LifeCore™ Biomedical, Inc. having the following specifications

Characteristics	Specification
Appearance	White to cream colored particles
Odor	No perceptible odor
Viscosity Average Molecular Weight	< 750,000 Daltons
UV/Vis Scan, 190-820nm	Matches reference scan
OD, 260nm	< 0.25 OD units
Hyaluronidase Sensitivity	Positive response
IR Scan	Matches reference
pH, 10mg/g solution	6.2 - 7.8
Water	8% maximum
Protein	< 0.3 mcg/mg NaHy
Acetate	< 10.0 mcg/mg NaHy

Heavy Metals, maximum ppm

As	Cd	Cr	Co	Cu	Fe	Pb	Hg	Ni
2.0	5.0	5.0	10.0	10.0	25.0	10.0	10.0	5.0

Microbial Bioburden	None observed
Endotoxin	< 0.07EU/mg NaHy
Biological Safety Testing	Passes Rabbit Ocular Toxicity Test

The following references teach hyaluronic acid, sources thereof and processes of the manufacture and recovery thereof.

United States Patent 4,141,973 teaches hyaluronic acid fractions (including sodium salts) having:

'(a) an average molecular weight greater than about 750,000, preferably greater than about 1,200,000 - that is, a limiting viscosity number greater than about 1400 cm^3/g., and preferably greater than about 2000 cm^3/g.;

(b) a protein content of less than 0.5% by weight;

(c) ultraviolet light absorbance of a 1% solution of sodium hyaluronate of less than 3.0 at 257 nanometers

wavelength and less than 2.0 at 280 nanometers wavelength;

(d) a kinematic viscosity of a 1% solution of sodium hyaluronate in physiological buffer greater than about 1000 centistokes, preferably greater than 10,000 centistokes;

(e) a molar optical rotation of a 0.1 - 0.2% sodium hyaluronate solution in physiological buffer of less than -11×10^3 degree - cm^2/mole (of disaccharide) measured at 220 nanometers;

(f) no significant cellular infiltration of the vitreous and anterior chamber, no flare in the aqueous humor, no haze or flare in the vitreous and no pathological changes to the cornea, lens, iris, retina, and choroid of the owl monkey eye when one milliliter of a 1% solution of sodium hyaluronate dissolved in physiological buffer is implanted in the vitreous replacing approximately one-half the existing liquid vitreous, said HUA being

(g) sterile and pyrogen free and

(h) non-antigenic.'

Canadian Letters Patent 1,205,031 (which refers to United States Patent 4,141,973 as prior art) refers to hyaluronic acid fractions having average molecular weights of from 50,000 to 100,000; 250,000 to 350,000; and 500,000 to 730,000 and discusses processes of their manufacture.

Where high molecular weight hyaluronic acid (or salts or other forms thereof) is used, it must be diluted to permit administration and ensure no intramuscular coagulation."

(v) and, at page 33, line 37 to page 35, line 30:

"Thus Applicant has combined hyaluronic acid (and sodium hyaluronate and/or other forms) with medicinal and/or therapeutic agents for the treatment of conditions and diseases with totally unexpected results:

For Example

Condition/Disease	Chemicals & Drugs
1. Cancer, increasing activity of macrophages	free radical scavenger, superoxide dismutase,

		ascorbic acid (Vitamin C) anti-cancer drugs, NSAID, Chemo-therapeutic Agents, detoxifying Agents (e.g. cholestyramine)
1A.	Reduction of swelling in brain of (DMSO)person suffering brain trauma	Dimethyl Sulfoxide
2.	Hair growth grow more hair when applied topically	minoxidil - combination
3.	Herpes, canker sore, shingles	nonionic surfactants, e.g., nonoxynol-9 and anionic, (e.g. cetyl pyridinium chloride) and cationic (e.g. benzalkonium chloride), surfactants
4.	Renal failure, cardiac insufficiency, hypertension, edema	diuretics - furosemide
5.	Infection, acne, mononucleosis	antibiotics, antibacterials, antimicrobials, etc., ascorbic acid and hyaluronic acid
6.	Transplants	cyclosporins
7.	Inflammation, elimination of tumor break down material	non-steroidal anti-inflammatories, NSAID e.g. (toxins and debris), diclofenac,
	decreasing side effects, relief of pain (e.g. back pain)	indomethacin, piroxicam, ibuprofen, tromethamine salt of Ketorolac, naproxen,
8.	Detoxification	enema, detoxifying agent, peritoneal dialysis
9.	Bronchodilation	bronchodilators, e.g. beclo-

		methasone diproprionate (sodium cromoglycate although not specifically a broncho-dialator), theophylline
10.	Vascular ischemia	treat limbs in respect of diabetes, Berger's disease, etc. with suitable medicine e.g. Trental
11.	HIV (AIDS)	DMSO, Vitamin C, NSAID (e.g. indomethacin, naproxen, ketorolac tromethamine), interferon, Vibramycin™, (doxycline), tetracycline
12.	Diabetes	insulin
13.	Post-menopause	estrogens replacement
14.	Prevention of topical infection	antimetabolites (e.g. sulfonamides)
15.	Reduction of swelling	DMSO
16.	Hypertension, cardiac	Calcium channel blockers e.g. insufficiency- Nifedipine ß-Blockers e.g. atenolol, propranolol
17.	Prostaglandin Synthesis inhibition	acetylsalicylic acid
18.	Enhance oxygenation of tissue by perfusion fluid bathing the tissue (for transplantation purposes"	perfusate

The above description and proposals will apply herein only modified to reflect the benefits of this invention. Thus, the administration of the form of hyaluronic acid and, medicine and/or therapeutic agent described above and identified in the Applications incorporated herein by reference may now be preceded by an effective amount (for example, exceeding in the order of about 3 - 5 mg/kg of body weight) of chondroitin sulphate (for example, 200 - 400 mg/70 kg person).

As a result, less medicine and/or therapeutic agent for example, a cytotoxic agent, and transport agent for example, hyaluronan, than would be normally or usually used for the treatment, may now be required because the hyaluronan together with the medicine and/or therapeutic agent now goes to the site in need of treatment (tumour, for example) and is not taken up by the liver which has now been "down regulated". Thus, the liver would not be as damaged by the for example, cytotoxic agent as in the past.

The chondroitin sulphate preferably may have a molecular weight exceeding 20,000 daltons for example, in the order of about between 20,000 and 40,000 daltons although there is a benefit irrespective of the molecular weight of chondroitin sulphate administered. Preferably, higher molecular weight chondroitin sulphate is used so long as it is in a dosage form that can be administered effectively (for example, in sufficient sterile water for intravenous purposes). The dextran sulphate or other agents (such as other glycosaminoglycans) may have a molecular weight for example, in the range between about 20,000 and 500,000 daltons or higher provided the dosages can be effectively administered.

Thus, according to one aspect of the invention, a method of treating a disease or condition in a human treatable by a medicine and/or therapeutic agent which may be transported by an agent (for example, a form of hyaluronic acid such as sodium hyaluronate) to the site in need of treatment in the body and which agent may also transport the medicine and/or therapeutic agent to the liver (by for example, the transport agent binding to receptors on the liver) is provided comprising:

(a) administering an effective amount of a first agent such as chondroitin sulphate which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver thereby "down regulating" the liver;

and,

(b) thereafter (for example, 3 to 4 minutes after the administration under sub-paragraph (a) of this paragraph) administering an effective non-toxic amount of a medicine and/or therapeutic agent and an effective amount of a second agent which is a transport agent [for the medicine and/or therapeutic agent] and

is a different agent from the first agent (for example, a form of hyaluronic acid) and which second agent binds to the site in need of treatment and would be capable of binding to the sites of the liver if the liver had not been "down regulated" so that the liver's binding capacity for the second agent has been substantially reduced (preferably substantially eliminated or blocked) by the up-take by the liver of the first agent (for example, chondroitin sulphate Molecular Weight 20,000) administered under sub-paragraph (a) by for example, binding with the scavenger receptors of the liver.

Because the liver has been "down regulated", the amounts of medicine and/or therapeutic agent that may be effective to treat the site in need of treatment is substantially reduced. As well, the amount of the for example, form of hyaluronic acid (transport agent) is substantially reduced so that the effective amount is substantially less than 10 mg/70 kg person (for example, 20 µg/kg of body weight of the patient being treated).

According to another aspect of the invention, a method of protecting the liver from taking up medicines and/or therapeutic agents toxic to the liver when administering the medicine and/or therapeutic agent to a site in need of treatment, is provided comprising:

(a) administering an effective amount of a first agent such as chondroitin sulphate which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver thereby "down regulating" the liver;

and,

(b) thereafter (for example, 3 to 4 minutes after the administration under sub-paragraph (a) of this paragraph) administering an effective non-toxic amount of a medicine and/or therapeutic agent and an effective amount of a second agent which is a transport agent and is a different agent from the first agent, (for example, a form of hyaluronic acid) and which second agent binds to the site in need of treatment and would be capable of binding to the sites of the liver if the liver

had not been "down regulated" so that its binding capacity for the second agent has been substantially reduced (preferably substantially eliminated or blocked) by the up-take by the liver of the first agent (for example, chondroitin sulphate, Molecular Weight 20,000) administered under sub-paragraph (a) by for example, binding with the scavenger receptors of the liver.

The amount of the first agent administered under (a) for example, in the order of at least about 3 - 5mg/kg of body weight (for example, 200 - 400 mg/70 kg person) having preferably a molecular weight in the range of 20,000 to 40,000 daltons, may be administered by any suitable manner such as systemically for example, orally, intravenously, subcutaneously or by direct injection proximate, adjacent, or into, the liver (by direct administration into the hepatic artery). Thereafter, (for example, after 3 to 4 minutes) the amount of the second agent for transport in sub-paragraph (b) for example, sodium hyaluronate having a molecular weight less than 750,000 daltons is administered in an effective amount now found to be substantially less than 10mg/70kg person discussed in WO 91/04058 together with the medicine and/or therapeutic agent. The at least 200 mg/70kg person of for example, the sodium hyaluronate provided in Application WO91/04058 to reduce the side effects of the medicine and/or therapeutic agent may now be substantially reduced because the amount of the medicine and/or therapeutic agent that is now effective is substantially less than previously provided. Thus, less of for example, the form of hyaluronic acid may now be administered in a dosage together with a lesser amount of what is now an effective amount of medicine and/or therapeutic agent to reduce the side effects of the medicine and/or therapeutic agent. Thus, the amounts of the medicine and/or therapeutic agent and hyaluronan transport agent may now be (µg) microgram amounts per kilogram of body weight to be effective. .

The first agent may be chondroitin sulphate (preferably) or other suitable agent (such as dextran sulphate or other GAGS [Glycosaminoglycans] and/or their proteoglycan forms which are not a form of hyaluronic acid). Other scavenger receptor ligands which are effective may also be used as the first agent. The second agent is preferably a form of hyaluronic acid such as hyaluronan or sodium hyaluronate.

Therefore, according to another aspect of the invention, I have provided a dosage kit for maximizing the amount of medicine and/or therapeutic agent to be delivered to a site in the body in need of treatment and/or for protecting the liver from taking up medicine and/or therapeutic agent (particularly cytotoxic agents) when medicines and/or therapeutic agents must be delivered to treat sites other than the liver, comprising:

(a) an effective dosage amount of a first agent such as chondroitin sulphate which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver to thereby "down regulate" the liver;

and, separately from the dosage amount of (a), and,

(b) an effective dosage amount comprising an effective non-toxic dosage amount of a medicine and/or therapeutic agent and an effective amount of a second agent which is a transport agent and is a different agent from the first agent (for example, a form of hyaluronic acid) and which second agent will bind to the site in need of treatment and would be capable of binding to the sites of the liver if the liver is not down regulated so that the liver's binding capacity for the second agent has been substantially reduced (preferably eliminated or blocked) by the up-take by the liver of the first agent (for example, chondroitin sulphate Molecular Weight 20,000) when administered after for example, at least about 3-4 minutes after administration of the dosage amount of the first agent under sub-paragraph (a) by for example, binding with the scavenger receptors of the liver.

The dosage amounts for sub-paragraph (b) may be microgram (μg) per kilogram of body weight for example, 20 μg/kg.

Further, according to another aspect of the invention a method is provided, comprising:

(a) administering an effective amount of a first agent which does not bind to receptors at the site in need of

treatment but which binds with receptors of the liver thereby "down regulating" the liver;

and,

(b) thereafter administering an effective amount of a medicine and/or therapeutic agent (for example, an NSAID [non-steroidal anti-inflammatory agent] or cytotoxic agent (for example, methotrexate and cisplatin and combinations thereof) and an effective amount of a second agent which is a transport agent and is a different agent from the first agent and which second agent is a transport agent which binds to the site in need of treatment and transports to the interstitial fluid, lymph and lymph nodes, and would be capable of binding to the sites of the liver if the liver had not been "down regulated" so that its binding capacity for the second agent has been substantially reduced by the up-take by the liver of the first agent administered under sub-paragraph (a) by binding with the scavenger receptors of the liver.

The first agent may be chondroitin sulphate and the second agent may be a form of hyaluronic acid. The amounts of each may be as previously discussed. For example, the amount of chondroitin sulphate may exceed at least about 3-5mg/kg and the effective amount of the form of hyaluronic acid may exceed 0.1 mg/70kg person and may have a molecular weight less than 750,000 daltons.

The invention will now be illustrated by reference to the following Figures and examples and discussion with respect thereto:

Figure 1 illustrates the Biodistribution of Labeled Hyaluronan, 18-20h after Intravenous Injection of 1 mg Chondroitin Sulphate Followed by 1 mg Labeled Hyaluronan;

Figure 2 illustrates the Uptake of 1 mg Labeled Hyaluronan (HA) With or Without Preinjection of 1 mg Chondroitin Sulphate (CS);

Figure 2b illustrates the Uptake of 1 mg Labeled Hyaluronan;

Figure 3 is made up of two drawings, the top drawing comparing MCPM/rat v. Time (min.), and the lower drawing comparing the MCPM/organ when 1 mg of chondroitin sulphate was administered followed by ^{125}I-HA (^{125}I-Hyaluronan).

Figure 4 illustrates the inhibition of labeled hyaluronan (HA) binding to NGW cells at 37°C. (The chondroitin sulphate does not interfere whereas the labeled hyaluronan does.)

Figure 5 shows in vivo images of the reduced liver uptake of labeled hyaluronan (HA) after pre-treatment of rats by chondroitin sulphate (CS).

Figure 6 illustrates the targeting of tumours by trace amount of labeled HA (hyaluronan) after administration of chondroitin sulphate (CS) in an effective amount of 200 - 400 mg/70 kg person of CS.

Figure 7 illustrates the photoimaging of clearance of ^{125}I-T-HA.

Figures 8 to 14 illustrate the presence of ratioactive HA in the body and its characteristics with or without prior administration of other agents.

A summary of the data I have developed on tumour targeting using radiolabelled hyaluronan (HA) administered after chondroitin sulphate (CS) pretreatment is set out in the Figures and is discussed below. I used female Wistar FU rats (weight in the order of 250 gm per rat on average) inoculated in one hind leg with a rat colon carcinoma called NGW to develop the data. When the tumours appeared, the rats received an intravenous injection of 1 mg chondroitin sulphate (200 - 400 mg/70 kg person) followed 30 seconds later by 1 mg HA (hyaluronan) of low specific radioactivity. 18 to 20 hours later the animals were sacrificed and the radioactivity in the organs measured (see Fig. 1).

I have with the same model used 1 mg HA (hyaluronan) of similar specific activity alone and found a tumour to non-tumour ratio of 7.79+5.00.

Using chondroitin sulphate (CS) pretreatment the ratio is increased to 16.23+2.48. The increase is mainly due to a lower uptake in the non-tumour tissue (muscle of the healthy leg) but there is also a 27% increase in total amount bound (see Fig. 2).

The individual values show that 4 out of 5 "controls" have a tumour to non-tumour ratio of about 4 (the relatively high ratio of 7.79 is due to one single experiment with a high ratio). In earlier published studies with labeled HA alone, I have seen tumour to non-tumour ratios of about 4 using similar, but not identical tumour systems. See "Accessible hyaluronan receptors identical to ICAM-1 in mouse mast

cells", Stefan Gustafson, et al., Glycoconjugate Journal (1995) 12:350-355. Therefore, I have now developed a real improvement using chondroitin sulphate (CS) to the delivery of medicine and/or therapeutic agents. The administration of chondroitin sulphate (CS) effectively blocks liver uptake of labeled HA (hyaluronan) at 10-15 minutes by about 80% (see Fig. 3) (without chondroitin sulphate (CS) the liver would have absorbed 95% of the radioactivity at this time).

In vitro data also developed by me establishes the NGW tumour cells have HA (hyaluronan) take-up receptors that are not inhibited (blocked/immobilized for a period of time from taking up the second agent (for example, hyaluronan)) by chondroitin sulphate (CS).

Figure 4 illustrates three determinations which clearly show that the uptake of radiolabelled hyaluronan is not interfered with by the chondroitin sulphate but is interfered with by the unlabeled hyaluronan.

Composite Figure 5 shows in vivo images of the reduced liver uptake of labeled HA after CS pre-treatment of rats. This figure shows that also uptake of trace amounts of HA (equivalent to 0.5-1 mg/70 kg person) can be effectively inhibited for an extended time by CS (equivalent dose 1-2 gm/70 kg person). Thus, one may use low amounts of not only active drug (medicine and/or therapeutic agent) (μg/kg) but also of HA (μg/kg of body weight of the patient). Thus, low amounts of HA (much less than 10 mg/70 kg person) can be used as the transport agent. Thus, I have developed a treatment whereby a low dose of HA and low dose of active drug (medicine and/or therapeutic agent) that really targets the site of treatment (for example diseased tissues) which is best has been provided and which also reduces side effects.

I have also performed experiments on tumour rats using 1 mg CS (200 - 400 mg /70 kg person) followed by a trace dose of labeled HA (equivalent to 0.5-1 mg/70kg person) and seen good targeting to the tumour (see Figure 6). The experimental conditions are identical to those previously described for NGW tumour rats, except for the low dose of HA. The relatively high radioactivity in the control muscle is most likely due to circulating degradation products at this time point (about 20 h after injection). Even so, the tumour to non-tumour ratio is 8.8 ($p<0.001$, $n=4$), showing that the targeting works also at lower HA doses.

Some preliminary size exclusion chromatographic studies were conducted on the Mw (molecular weight) distribution of radiolabeled HA

(hyaluronan) in serum and urine of rats receiving a pretreatment of 5mg CS and then 5 μg 125I-HA (hyaluronan) with a molecular weight of about 400 kDa (400,000 daltons). Blood was collected at 2-4, 10-12, 22-24 and 70 minutes after the completion of the administration. Urine was collected from the bladder at 70 minutes when the animals were killed and organs collected. The results show that the Mw (molecular weight) of hyaluronan goes down (reduces in the body) by time and at 70 minutes, most of the hyaluronan that is left in the circulation has a molecular weight less than 39 kDa (39,000 daltons). At this time, about 10% of the injected radioactivity was found in the urine and had a mean Mw (molecular weight) of about 20 kDa (20,000 daltons). The radioactivity in the liver was only 6-7% while kidneys contained about 9%, I believe, due to material being filtered out into urine. Blood contained around 35% and as other organs contained only negligible amounts, about 40% has been filtered out into peripheral tissues. The chondroitin sulphate (CS) blocking is therefore an ideal way of getting some hyaluronan out into the tissues. This is an additional factor in the increased binding of intravenously administered hyaluronan (HA) to tumour tissue that I found using chondroitin sulphate pretreatment. This provides a further method of delivering a form of hyaluronan (HA) together with a medicine and/or therapeutic agent into interstitial fluid, lymph and lymph nodes for the treatment of disease for example, cancer and metastases. This treatment may also be used to prevent metastases.

Hyaluronan (hyaluronic acid; HA) is a high molecular weight polysaccharide consisting of repeating units of glucuronic acid and N-acetylglucosamine. It is found in high concentrations in connective tissues such as skin and cartilage, in the vitreous body of the eye and in synovial fluid (1). The polysaccharide can associate with several proteins in the extracellular matrix and also with some cell-surface HA-binding proteins (2).

The serum level of HA is normally very low (10-50μg/l), but elevated in certain disease states such as rheumatoid arthritis, liver cirrhosis, and various malignancies (3). Circulating hyaluronan comes from the peripheral tissues where most is associated with cells or binding proteins, but some exists in freely mobilized compartments. The polysaccharide enters the general circulation via the lymph (4) after 80-90% is removed in lymph nodes before reaching the bloodstream (5). The

Mw in serum is in the order of $1.5x10^5$ while the Mw of HA in lymph is about $2x10^6$ (6). The major site for elimination of HA from the bloodstream, under normal circumstances, is via receptor mediated endocytosis by the liver (1,7). The $t_{1/2}$ of intravenously administered hyaluronan to experimental animals and man is in the order of a few minutes, and already after 15-20 min the degradation products start to appear in the circulation (7-9). The uptake is via coated pits and coated vesicles in liver endothelial cells (LEC), while Kupffer cells and hepatocytes are essentially negative for uptake both *in vivo* and *in vitro* (9-11).

The HA taken up by LEC is transported to lysosomes where it is degraded to monosaccharides that ultimately are broken down to carbon dioxide, urea and water in the hepatocytes (12).

Inhibition studies with LEC in culture show that the receptors recognise other ligands besides HA, such as chondroitin sulphate (CS), dextran sulphate (DxS) and desulphated CS (13-15). We have now performed inhibition studies with polysaccharides that might bind to the HA receptor on LEC *in vivo*, and can in the present paper show that CS and DxS, but not heparin, inhibit the clearance of HA by the liver. The HA that remains in the circulation is broken down to smaller fragments by what seems to be a specific and saturable mechanism, as high concentrations of HA inhibit this degradation. This mechanism could explain why circulating HA has a much lower Mw than the HA entering the general circulation via the lymph. Saturation of this mechanism could also explain why extremely high circulating levels of HA can give rise to increased plasma viscosity from high Mw HA.

Materials and methods

Polysaccharides: The HA used for labelling and uptake- and turnover-studies was supplied by Hyal Pharmaceutical Corporation (HPC), Toronto, Canada. The molecular weight distribution of the HA was determined by chromatography on a calibrated column of Sephacryl HR with porosities noted as 400, 1000 and 2000 (Pharmacia, Uppsala, Sweden) in 0.25M NaCl, 0.05% chlorbutanol (16). The HA content in each fraction was monitored by determination of the absorbance at 214 nm. Radioactivity was measured by gamma-counting on a Packard auto-gamma gamma-counter. Chondroitin sulphate A from bovine trachea was from Sigma chemical

company, St. Louis, U.S.A. (product number 8529). This batch contained 1.9 ng HA/µg CS as determined by a specific radioassay for HA (HA-50, Pharmacia, Upssala, Sweden).

Dextran sulphate with a Mw of approximately 500,000 Da was from Pharmacia Biotech, Uppsala, Sweden (Code No. 17-0340-01).

Heparin from intestinal mucosa and purified by repeated precipitation with cetylpyridinium chloride (17) was a kind gift from professor Ulf Lindahl, University of Uppsala, Sweden.

Labelling of HA, CS and heparin: The HA, CS and heparin was labelled with DL-tyrosine (Sigma chemical company St. Louis, U.S.A.) as previously described (18), after CNBr- activation of the polysaccharide. Briefly, 15mg HA, CS or heparin was activated by pH 11 by 8mg CNBr for 5 min. The activated polysaccharide was separated from the reaction mixture on a small column of Sephadex G25 (PD 10, Pharmacia, Uppsala, Sweden) equilibrated with 0.2M borate buffer pH 8.0. The activated polysaccharide was incubated over night with 1 mg tyrosine (T) (Sigma chemical company, St. Louis, U.S.A.). The T bound to HA (T-HA), CS (T-CS) or heparin (T-Hep) was separated from unbound T on a PD 10 column equilibrated with phosphate buffered saline (pH 7,5) (PBS), containing NaCl (8 g/l), KCl (0,2 g/l), KH_2PO_4 (0,2 g/l) and Na_2HPO_4 (1,15 g/l).

A part of the T-HA, T-CS or T-Hep was iodinated with ^{125}I by placing 100µg xof T-labelled polysaccharide together with 0.5 mCi ^{125}I in a small glass tube covered with a film of 10µg 1,3,4,6-tetrachloro-3a,6a-diphenylglycouril (Sigma chemical company, St. Louis, U.S.A.). Unincorporated ^{125}I was removed on a PD 10 column equilibrated with PBS and the iodinated T-HA (^{125}I-T-HA), T-CS (^{125}I-T-CS) or heparin (^{125}I-T-Hep) stored at 5°C. The specific radioactivity was usually 1500-5000 dpm/ng.

The ^{125}I-T-HA kept a high molecular weight-profile upon gel filtration chromatography with a mean Mw of around $0.5x10^6$ Da, and was found to be cleared from the circulation with the kinetics and organ distribution reported for biosynthetically labelled HA of high Mw. The ^{125}I- labelled T-HA was also taken up by isolated rat liver endothelial cells both *in vivo* and *in vitro*, indicating that the labelling does not interfere with the binding to specific cell-surface receptors found on these cells (1, 2, 9-11).

The ^{125}I-T-CS and ^{125}-T-Hep was, by gel filtration chromatography on Sephacryl S-1000 and S-300 calibrated with HA standards, found to have

the same mean Mw as the unlabelled CS (approximately 30,000 Da) and heparin (approximately 20,000 Da) and showed similar size distribution patterns.

Cells: A single cell suspension was prepared from the liver of Sprague Dawley rats, weighing 200-300g, by collagenase perfusion for 10 minutes at 37°C. Liver endothelial cells, Kupffer cells and parenchymal cells were purified by Percoll®-centrifugation and selective adherance as described by Pertoft and Smedsrød (19), giving approximately 95% pure cells (10, 19). Monolayer cultures were maintained under standard culturing conditions in RPMI medium supplemented with L-glutamine (2 mM), gentamicin (50µg/ml) and, in the case of parenchymal cells, 10% (v/v) fetal calf serum. Liver endothelial cells were cultured entirely without serum. All cells were cultivated overnight before the start of the experiments.

Uptake studies with cells in culture: ^{125}I-T-hyaluronan, and in competition experiments unlabelled polysaccharides, were added to cold RPMI medium containing L-glutamine (2 mM) and gentamicin (50µg/ml), and given to cultures of 100,000-200,000 liver endothelial cells in fibronectin-coated dishes with a diameter of 16mm. The cultures were kept under standard culturing conditions in 300ml medium.

After the termination of the incubations, the medium was removed and analyzed for radioactivity, thereafter it was in some experiments subjected to gel cromatography on a 24ml Sephacryl 300 column to separate degraded from undegraded polysaccharide. The cells washed three times in phosphate buffered saline (pH 7,5)(PBS), containing NaCl (8g/l), KCl (0,2 g/l), KH_2PO_4 (0,2 g/l) and Na_2HPO_4 (1,15 g/l), analyzed for radioactivity, or homogenized and fractionated as described earlier (18). Unspecific binding was corrected for by measurement of radioactivity associated to dishes without cells, which generally was just above background levels.

In vivo studies: Sprague Dawley rats, weighing 200-300g, were anesthetized with pentobarbital (45mg/kg body weight). They received an injection in the tail vein of 5µg ^{125}I-T-hyaluronan or ^{125}I-T-Hep (8-15x10^6 cpm), in 0.8-1.0 ml 0.15 M NaCl, 10 mM NaH_2PO_4, pH 7.4. In some studies the rats received 1-5mg unlabelled polysaccharides 30 seconds prior to the labelled polysaccharide. Blood samples were repeatedly collected from the distal part of the tail during the circulation period. In some cases serum was subjected to size exclusion chromatography on a Sephacryl S 300 and

the radioactivity of the eluate analysed.

After 10min-22h the rat was killed. Liver, lungs, kidneys, heart, spleen and in some instances urine were assayed for radioactivity. The data were processed using a Macintosh SE/30®, Macintosh IIsi® or Macintosh 7200 computer (Apple computer Inc. Cupertino, CA, U.S.A.). The graphs were constructed using the Cricket Graph® program (version 1.3, Cricket software, Malvern, PA, U.S.A.) and Canvas (verson 3.0.2, Deneba Systems Inc., Miami, Fl, U.S.A.). Statistical analysis was performed using Statworks® (version 1.1, Cricket Software, Malvern, PA, U.S.A.).

Scintigraphic studies: The rats were anesthetized and injected as described above. In dynamic studies the injections were made with the rats placed on a Fuji phosphoimager screen with a high resolution brass collimator between rat and screen. The screen was exposed for 10 min. and the image developed and analyzed on a Fuji 2000 phospho imager. Some image analysis studies were performed using the NIH Image software.

Results

A tracer dose of intravenously administered ^{125}I-T-HA was by phosphoimaging shown to be rapidly cleared by the rat liver (Fig. 7). A small amount of radioactivity, attributable to small amounts of ^{125}I-T lost from the labelled polysaccharide, could be visualized in the urinary bladder (Fig. 7). During 22h, approximately 20% of the radioactivity disappeared from the liver and could be found in the urine (bedding in the cage).

When CS, at a dose of about 20mg/kg body weight, was administered prior to ^{125}I-T-HA the rapid clearance was lost and the major part of radioactivity could be visualized scattered over the entire animal for hours (Fig. 7). The radioactivity after CS blocking was mainly found in the blood with some uptake in liver, spleen and kidney (Fig. 8) and was found to rapidly decrease in Mw (Fig. 9). Some labelled polysaccharides with Mw of about 10,000-40,000 Da were found in urine (Fig. 8 and 9). Only minute amounts of labelled HA could be found in the urine when liver uptake was blocked by unlabelled HA (1 mg/kg b.w.) (Fig. 8). The rapid decrease in Mw of circulating ^{125}I-T-HYA seen after CS blocking was not seen with HA blocking and more radioactivity stayed within the general circulation after HA blocking than after CS blocking (Fig. 8 and Fig. 10). However, trace amounts of radioactivity could be found in the urine after 70 min., this material had the same Mw as the radioactivity found in urine after CS

blocking (Figs. 8, 9 and 10).

DxS (Dextran Sulphate) was found to effectively block liver uptake at a dose of 200mg/kg b.w., and result in increased outflow of labelled HA from the general circulation as seen by low liver uptake and low recovery of injected HA (Fig. 11). When a dose of 1mg/kg bw. was tested it was found that the liver uptake was inhibited by 30-40% (Fig. 11).

Heparin at a dose of 20mg/kg b.w. did not affect the clearance of ^{125}I-T-HYA (Fig. 8), nor could HA at a dose of 20mg/kg b.w. inhibit the clearance of ^{125}I-T-Hep at a tracer dose (Fig. 12). CS could partially inhibit the liver uptake of ^{125}I-T-Hep (Fig. 12).

The binding of ^{125}I-T-HYA to LEC in culture was effectively inhibited by HA, CS and DxS (Fig. 13).

When the biodistribution of intravenous ^{125}I-T-CS was studied it was found that the liver uptake was lower than for ^{125}I-T-HYA, as was the total recovery of radioactivity, while the urinary excretion was high (Fig. 14). The liver uptake could effectively be inhibited by unlabelled CS and HA, resulting in increased urinary clearance (Fig. 14).

Discussion

That the receptor mediated endocytosis of HA by LEC is not specific for HA has earlier been shown by experiments with isolated LEC in culture (13-15). In such studies the receptors also recognise ligands such as CS and DxS. The present investigation was performed in order to see if some negatively charged polysaccharides will influence the turnover of circulating HA *in vivo*. We have used a labelling technique that does not alter the Mw nor interfere with the cell binding properties of polysaccharides and results in a derivative with g-radiation of high specific activity (18, 20-23). Such a polysaccharide is advantageous in many respects eg. it is easy to detect in low amounts and the distribution can be recorded in the live animal using scintigraphic or phosphoimaging techniques. We choose the rat for these studies as many turnover and uptake studies have earlier been performed with this species and normal blood levels and estimated turnover rates are similar to the ones found in man (1, 3, 9, 10, 24).

Our studies show that CS and DxS, but not heparin, inhibit the clearance of HA from the bloodstream via inhibition of the receptor mediated endocytosis by the liver (Fig. 7 and 11). Heparin seems to be cleared by mechanisms not affected by HA, while CS seems to partially reduce the

liver uptake of labelled heparin (Fig. 12), but the inhibition is only partial and not as effective as the blocking of labelled HA. As a result of CS or DxS blocking of liver HA-receptors, the HA that remains in the cirulation is rapidly broken down to smaller fragments by what seems to be a specific and saturable mechanism, as high concentrations of HA inhibits this degradation (Figs. 9 and 10). The fragmentation results in low recovery of injected dose and the low Mw HA is filtered out into the tissues and via the kidneys out into the urine (Fig. 7, 9 and 10). We have done the size determination of circulating material by size exclusion chromatography of serum or plasma on a column of Sephacryl S 300. This gel will not separate HA above 50 kDa very well so it is possible that the breakdown of the injected material with a Mw of about 400 kDa is broken down by shear forces to a Mw above 50 kDa when unlabelled HA is given to reduce liver uptake. However, the breakdown to smaller fragments, resulting in the appearance of material chromatographing at an included position, occurs early after injection only in the case of CS blocking and not to any great extent when unlabelled HA is used as blocking agent (Figs. 9 and 10).

That CS and HA are recognized by the same receptors in the liver is also shown by the fact that not only unlabelled CS but also HA can inhibit the liver uptake of ^{125}I-T-CS (Fig. 14). That the liver uptake of CS is not as high as that of HA probably depends on the fact that the CS used only has a Mw of around 30 kDa compared to about 400 kDa for the HA, and some is therefore rapidly removed from the circulation by filtration so that only a fraction of that injected remains long enough in the general circulation to be taken up by the liver.

DxS seems also to bind to the same receptors as CS and HA but with lower affinity as a higher dose is needed to inhibit liver uptake of ^{125}I-T-HA using DxS than using CS or HA (Fig. 11).

Our results indicate that the turnovers of the naturally occuring polysaccharides HA and CS are partially an effect of liver uptake of the circulating polysaccharides via a common receptor on LEC. It is therefore possible, that high levels of circulating HA in some conditions can be secondary to increased outflow of CS into the general circulation from the tissues, and vice versa. The present results also argue, due to the presence of a more specific degradative mechanism of HA in the circulation, that if the LEC are blocked by CS, the urinary excretion of HA should be grossly increased in relation to the effect on serum levels, while increases in

serum levels of HA due to increased outflow of HA into the circulation or decreasd clearance by the liver, would result in only moderate increases in urinary excretion. Such a lack of correlation between serum levels and urinary excretion has been described earlier in a study of rheumatoid arthritis (RA), primary biliary cirrhosis (PBC) and Werners syndrome (25). All three diseases cause increased serum levels of HA. However, the urinary excretion in RA and PBC is only slightly increased, whereas a tenfold increase in excretion is seen in Werners syndrome, despite the fact that the serum level in this disease was lower than for PBC and RA.

The presence of a saturable degradative mechanism for HA in the circulation could also explain why circulating HA has a much lower Mw than the HA entering the general circulation via the lymph (6, 26). Size reduction of the polymer by mechanical shearing has been suggested as a mechanism by Fraser (26). However, the Mw and concentration of the unlabelled HA used to block liver uptake in the present study makes it unlikely that the viscosity was influenced to such an extent that breakdown by shearing would be inhibited. Fraser has stated that degradation by serum hyaluronidase does not occur under physiological conditions, that free radical activity in serum would be too low to cause the size reduction and that the degradation of high Mw HA is not inhibited by high doses of low Mw HA. The present study also argues against free radical attack as the degradation occurs in the presence of a high dose of CS that probably would scavenge any active free radicals. The site of degradation could be via a hyaluronidase, fixed on a cell surface and with a Mw-dependent binding of HA necessary for activity. Further studies are needed to characterize this mechanism that might prove to be an important part of HA metabolism.

As many changes can be made to the embodiments without departing from the scope of the invention, it is intended that all material contained herein be interpreted as illustrative of the invention and not in a limiting sense.

THE EMBODIMENTS OF THE INVENTION FROM WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE AS FOLLOWS:

1. A method of treating a disease or condition in a human treatable by a medicine and/or therapeutic agent which may be transported by an agent to the site in need of treatment in the body and which agent may also transport the medicine and/or therapeutic agent to the liver (by for example, the transport agent binding to receptors on the liver) comprising:

(a) administering an effective non-toxic amount of a first agent which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver thereby "down regulating" the liver;

and,

(b) thereafter administering an effective non-toxic amount of a medicine and/or therapeutic agent and an effective amount of a second agent which is a transport agent and is a different agent from the first agent and which second agent binds to the site in need of treatment and would be capable of binding to the sites of the liver if the liver had not been "down regulated" so that its binding capacity for the second agent has been substantially reduced by the up-take by the liver of the first agent administered under sub-paragraph (a) by for example, binding with the scavenger receptors of the liver.

2. The method of Claim 1 wherein the first agent is a glycosaminoglycan which is not a form of hyaluronic acid.

3. The method of Claims 1 or 2 wherein the first agent is chondroitin sulphate.

4. The method of Claims 1, 2, or 3 wherein the second agent (transport agent) is selected from a form of hyaluronic acid.

5. The method of claims 1, 2, 3, or 4 wherein the form of hyaluronic acid is selected from the group consisting of hyaluronic acid (hyaluronan) and a pharmaceutically acceptable salt thereof.

6. The method of Claims 3 or 4 wherein the amount of chondroitin sulphate exceeds about 3 - 5mg./kg of the human.

7. The method of Claims 6 wherein the second agent is an effective amount of the form of hyaluronic acid having a molecular weight less than 750,000 daltons.

8. A method of protecting the liver from taking up medicines and/or therapeutic agents toxic to the liver comprising:

(a) administering an effective amount of a first agent which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver thereby "down regulating" the liver;

and,

(b) thereafter administering an effective amount of a medicine and/or therapeutic agent and an effective amount of a second agent which is a transport agent and is a different agent from the first agent and which second agent is a transport agent which binds to the site in need of treatment and would be capable of binding to the sites of the liver if the liver had not been "down regulated" so that its binding capacity for the second agent has been substantially reduced by the up-take by the liver of the first agent administered under sub-paragraph (a) by binding with the scavenger receptors of the liver.

9. The method of Claim 8 wherein the first agent is chondroitin sulphate and the second agent is a form of hyaluronic acid.

10. The method of Claim 9 wherein the form of hyaluronic acid is selected from the group consisting of hyaluronic acid and pharmaceutically acceptable salts thereof.

11. The method of Claim 10 wherein the amount of chondroitin sulphate exceeds 3 - 5 mg./kg.

12. The method of Claim 11 wherein the effective amount of the form of hyaluronic acid exceeds .1 mg./70 kg. person.

13. The method of Claim 11 or 12 wherein the effective amount of the form of hyaluronic acid has a molecular weight less than 750,000 daltons.

14. A dosage kit for maximizing the amount of medicine and/or therapeutic agent to be delivered to a site in the body in need of treatment and/or for protecting the liver from taking up a medicine and/or therapeutic agent when medicines and/or therapeutic agents must be delivered to treat sites other than the liver, comprising:

(a) an effective dosage amount of a first agent which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver to thereby "down regulate" the liver;

(b) an effective dosage amount comprising an effective non-toxic amount of a medicine and/or therapeutic agent and an effective amount of a second agent which is a transport agent and is a different agent from the first agent and which second agent binds to the site in need of treatment and would be capable of binding to the sites of the liver if the liver is not down regulated so that its binding capacity for the second agent has been substantially reduced by the up-take by the liver of the first agent when the second agent is administered after the dosage amount under sub-paragraph (a) is administered.

15. The dosage kit of Claim 14 wherein the first agent is chondroitin sulphate and the second agent is a form of hyaluronic acid.

16. The dosage kit of Claim 15 wherein the form of hyaluronic acid is selected from the group consisting of hyaluronic acid and pharmaceutically acceptable salts thereof.

17. The dosage kit of Claim 16 wherein the amount of chondroitin sulphate exceeds about 3 - 5 mg./kg. of the body.

18. The dosage kit of Claim 17 wherein the amount of the form of hyaluronic acid exceeds 0.1 mg./70 kg. person.

19. The dosage kit of Claim 17 or 18 wherein the form of hyaluronic acid has a molecular weight less than 750,000 daltons.

20. A method of treating a disease or condition in a human treatable by a medicine and/or therapeutic agent which may be transported by an agent to the site in need of treatment in the body and which agent may also transport the medicine and/or therapeutic agent to the liver (by for example, the transport agent binding to receptors on the liver) comprising:

(a) administering an effective non-toxic amount of a first agent which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver thereby "down regulating" the liver;

and,

(b) thereafter administering an effective non-toxic amount of a medicine and/or therapeutic agent which is substantially less than the amount normally considered effective and an effective amount of a

second agent which is a transport agent and is a different agent from the first agent and which second agent would bind to the site in need of treatment and would be capable of binding to the sites of the liver if the liver had not been "down regulated" so that its binding capacity for the second agent has been substantially reduced by the up-take by the liver of the first agent administered under sub-paragraph (a) by for example, binding with the scavenger receptors of the liver and which effective non-toxic amount of the second agent is in an effective amount which is substantially less than the amount normally considered effective.

21. The method of Claim 20 wherein the first agent is a glycosaminoglycan which is not a form of hyaluronic acid.

22. The method of Claims 20 or 21 wherein the first agent is chondroitin sulphate.

23. The method of Claims 20, 21, or 22 wherein the second agent (transport agent) is selected from a form of hyaluronic acid.

24. The method of claims 20, 21, 22, or 23 wherein the form of hyaluronic acid is selected from the group consisting of hyaluronic acid (hyaluronan) and a pharmaceutically acceptable salt thereof.

25. The method of Claims 22 or 23 wherein the amount of chondroitin sulphate exceeds about 3 - 5mg./kg of the human.

26. The method of Claim 25 wherein the second agent is an effective amount exceeding about 20 μg/kg of body weight of the patient of the form of hyaluronic acid having a molecular weight less than 750,000 daltons.

27. A method of protecting the liver from taking up medicines and/or therapeutic agents toxic to the liver comprising:

(a) administering an effective amount of a first agent which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver thereby "down regulating" the liver;

and,

(b) thereafter administering an effective amount of a medicine and/or therapeutic agent which is substantially less than the amount normally used and an effective amount of a second agent which is a transport agent and is a different agent from the first agent and which second agent is a transport agent which binds to the site in need of treatment and would be capable of binding to the sites of the liver if the liver had not been "down regulated" so that its binding capacity for the second agent has been substantially reduced by the up-take by the liver of the first agent administered under sub-paragraph (a) by binding with

the scavenger receptors of the liver and which effective amount of the second agent is in an effective amount which is substantially less than the amount normally considered effective.

28. The method of Claim 27 wherein the first agent is chondroitin sulphate and the second agent is a form of hyaluronic acid.

29. The method of Claim 28 wherein the form of hyaluronic acid is selected from the group consisting of hyaluronic acid and pharmaceutically acceptable salts thereof.

30. The method of Claim 29 wherein the amount of chondroitin sulphate exceeds 3 - 5 mg./kg.

31. The method of Claim 30 wherein the effective amount of the form of hyaluronic acid exceeds 0.1 mg./70 kg. person.

32. The method of Claim 30 wherein the effective amount of the form of hyaluronic acid has a molecular weight less than 750,000 daltons and is an amount exceeding about 20 µg/kg of body weight of a patient.

33. A dosage kit for maximizing the amount of medicine and/or therapeutic agent to be delivered to a site in the body in need of treatment and/or for protecting the liver from taking up a medicine and/or therapeutic agent when medicines and/or therapeutic agents must be delivered to treat sites other than the liver, comprising:

(a) an effective dosage amount of a first agent which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver to thereby "down regulate" the liver;

(b) an effective dosage amount comprising an effective non-toxic amount of a medicine and/or therapeutic agent which is substantially less than the amount normally considered effective and an effective amount of a second agent which is a transport agent and is a different agent from the first agent and which second agent would bind to the site in need of treatment and would be capable of binding to the sites of the liver if the liver is not down regulated so that its binding capacity for the second agent has been substantially reduced by the up-take by the liver of the first agent when administered after the dosage amount under sub-paragraph (a) is administered and which effective non-toxic amount of the second agent is in an effective amount which is substantially less than the amount normally considered effective.

34. The dosage kit of Claim 33 wherein the first agent is chondroitin sulphate and the second agent is a form of hyaluronic acid.

35. The dosage kit of Claim 34 wherein the form of hyaluronic acid is selected from the group consisting of hyaluronic acid and pharmaceutically acceptable salts thereof.

36. The dosage kit of Claim 35 wherein the amount of chondroitin sulphate exceeds about 3 - 5 mg./kg.

37. The dosage kit of Claim 36 wherein the amount of the form of hyaluronic acid exceeds 0.1 mg./70 kg. person.

38. The dosage kit of Claim 36 wherein the effective amount of the form of hyaluronic acid has a molecular weight less than 750,000 daltons and is in an amount exceeding about 20 μg/kg. of body weight of a patient.

39. A method of preventing metastases in a person suffering from cancer comprising:

(a) administering an effective amount of a first agent which does not bind to receptors at the site in need of treatment but which binds with receptors of the liver thereby "down regulating" the liver;

and,

(b) thereafter administering an effective amount of a medicine and/or therapeutic agent and an effective amount of a second agent which is a transport agent and is a different agent from the first agent and which second agent is a transport agent which binds to the site in need of treatment and transports to the interstitial fluid, lymph and nodes and would be capable of binding to the sites of the liver if the liver had not been "down regulated" so that its binding

capacity for the second agent has been substantially reduced by the up-take by the liver of the first agent administered under sub-paragraph (a) by binding with the scavenger receptors of the liver.

40. The method of Claim 39 wherein the first agent is chondroitin sulphate and the second agent is a form of hyaluronic acid.

41. The method of Claim 40 wherein the form of hyaluronic acid is selected from the group consisting of hyaluronic acid and pharmaceutically acceptable salts thereof.

42. The method of Claim 41 wherein the amount of chondroitin sulphate exceeds 3 - 5 mg./kg.

43. The method of Claim 42 wherein the effective amount of the form of hyaluronic acid exceeds .1 mg./70 kg. person.

44. The method of Claim 42 or 43 wherein the effective amount of the form of hyaluronic acid has a molecular weight less than 750,000 daltons.

45. The method of Claim 39, 40, 41, 42, 43 or 44 wherein the medicine and/or therapeutic agent is selected from the group consisting of an NSAID and a cytotoxic agent and combinations thereof.

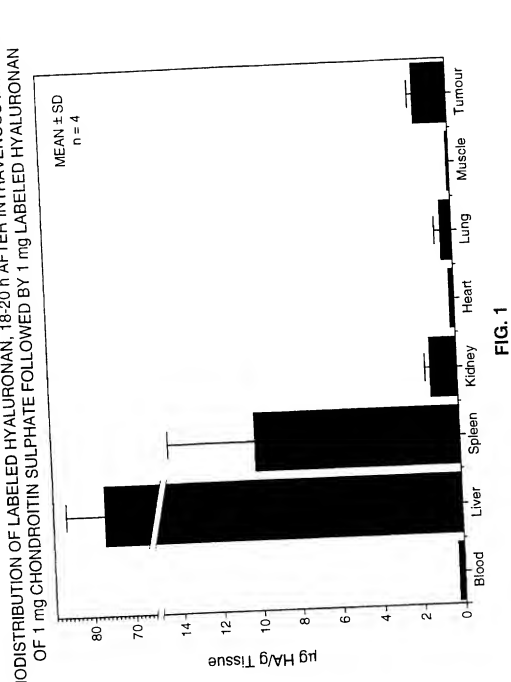

BIODISTRIBUTION OF LABELED HYALURONAN, 18-20 h AFTER INTRAVENOUS INJECTION
OF 1 mg CHONDROITIN SULPHATE FOLLOWED BY 1 mg LABELED HYALURONAN
MEAN ± SD
n = 4
µg HA/g Tissue
0
2
4
6
8
10
12
14
70
80
Blood
Liver
Spleen
Kidney
Heart
Lung
Muscle
Tumour

FIG. 1

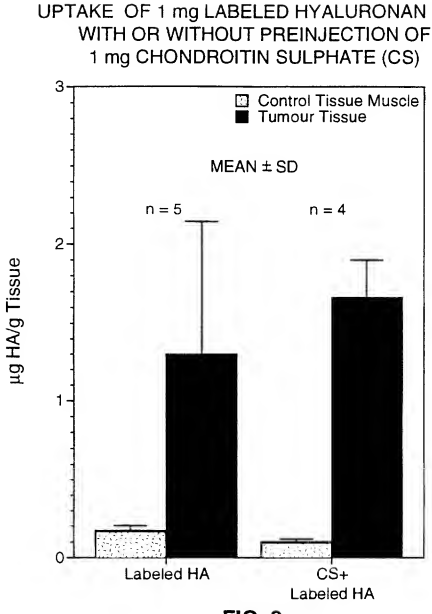

UPTAKE OF 1 mg LABELED HYALURONAN (HA)
WITH OR WITHOUT PREINJECTION OF
1 mg CHONDROITIN SULPHATE (CS)
Control Tissue Muscle
Tumour Tissue
MEAN ± SD
n = 5
n = 4
µg HA/g Tissue
3
2
1
0
Labeled HA
CS+
Labeled HA

FIG. 2

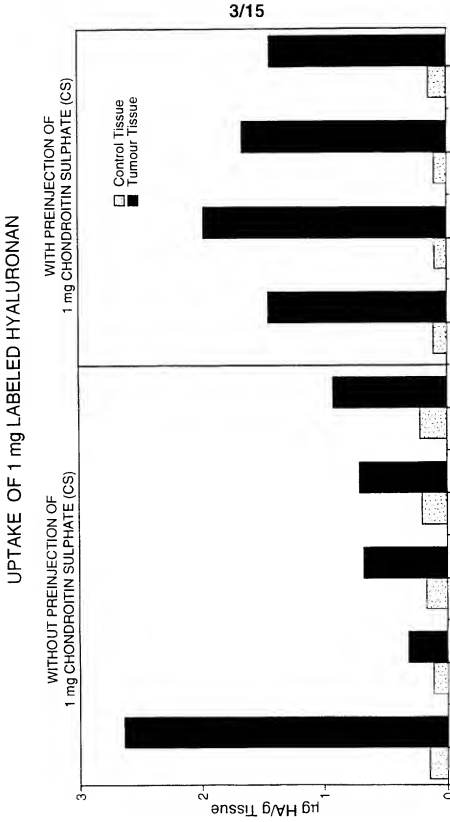
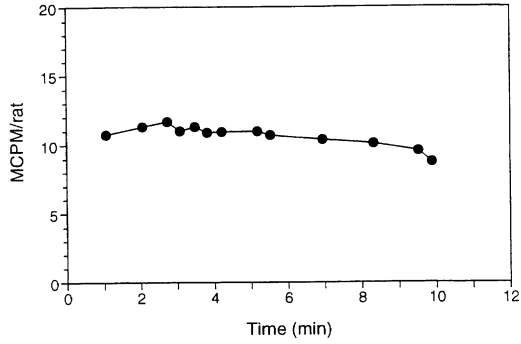

UPTAKE OF 1 mg LABELED HYALURONAN
WITHOUT PREINJECTION OF
1 mg CHONDROITIN SULPHATE (CS)
WITH PREINJECTION OF
1 mg CHONDROITIN SULPHATE (CS)
Control Tissue
Tumour Tissue
µg HA/g Tissue
0
1
2
3
1
2
3
4
5
1
2
3
4
RAT#

FIG. 2b


20
15
10
5
0
MCPM/rat
0
2
4
6
8
10
12
Time (min)

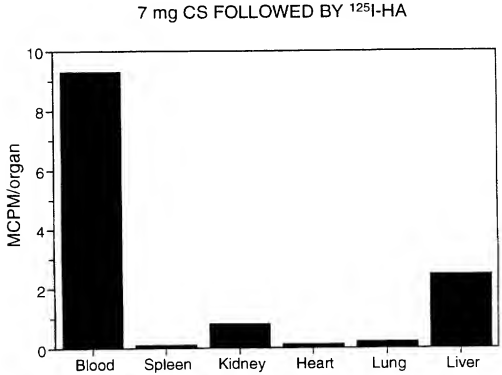

7 mg CS FOLLOWED BY 125I-HA
10
8
6
4
2
0
MCPM/organ
Blood
Spleen
Kidney
Heart
Lung
Liver

FIG. 3

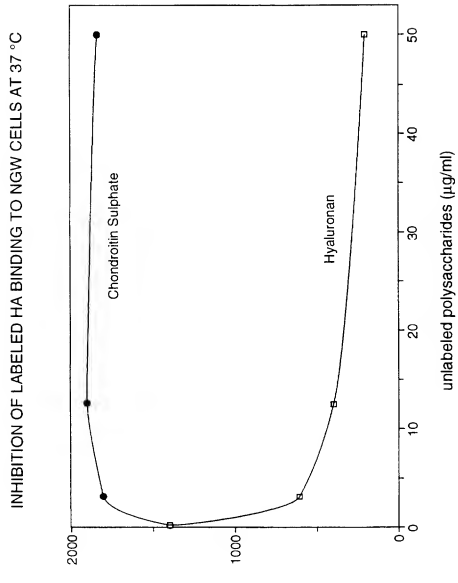

INHIBITION OF LABELED HA BINDING TO NGW CELLS AT 37 °C
cell associated radioactivity (cpm/dish)
2000
1000
0
0
10
20
30
40
50
unlabeled polysaccharides (µg/ml)
Chondroitin Sulphate
Hyaluronan

FIG. 4

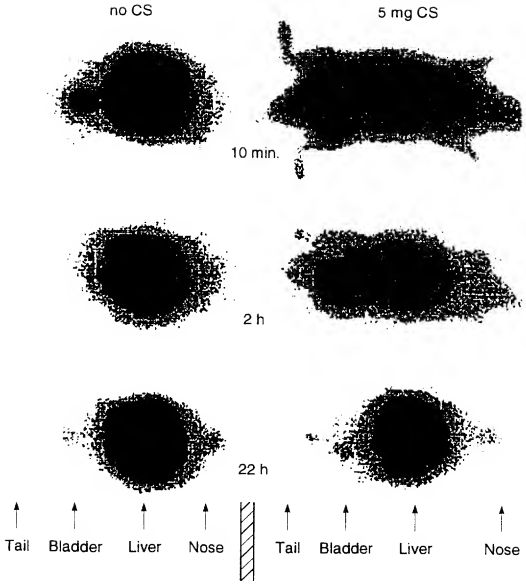

no CS
5 mg CS
10 min.
2 h
22 h
Tail
Bladder
Liver
Nose
Tail
Bladder
Liver
Nose

FIG. 5

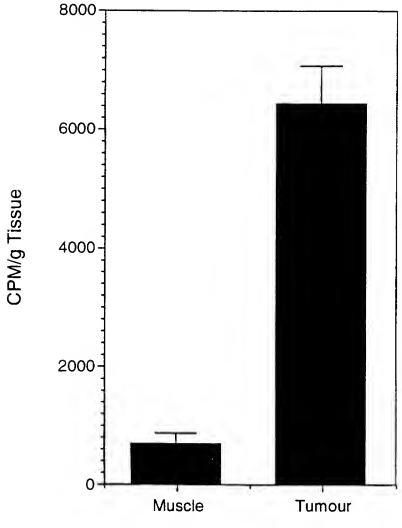


FIG. 6

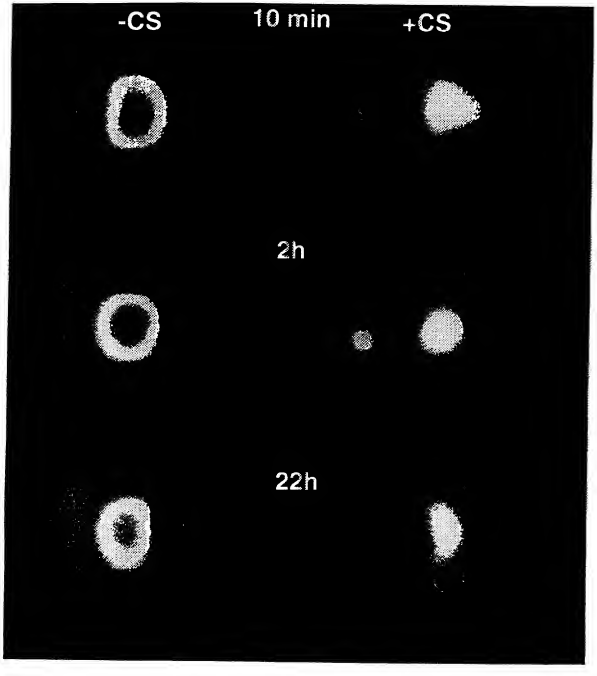


Fig. 7

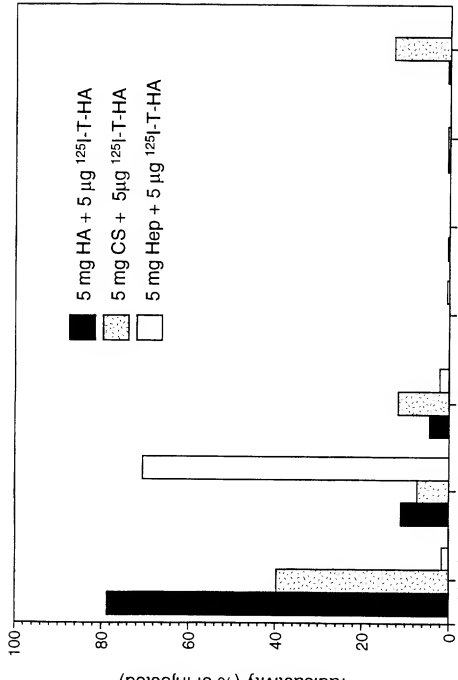

radioactivity (% of injected)
100
80
60
40
20
0
Blood
Liver
Kidney
Spleen
Heart
Lung
Urine
5 mg HA + 5 µg 125I-T-HA
5 mg CS + 5µg 125I-T-HA
5 mg Hep + 5 µg 125I-T-HA

FIG. 8

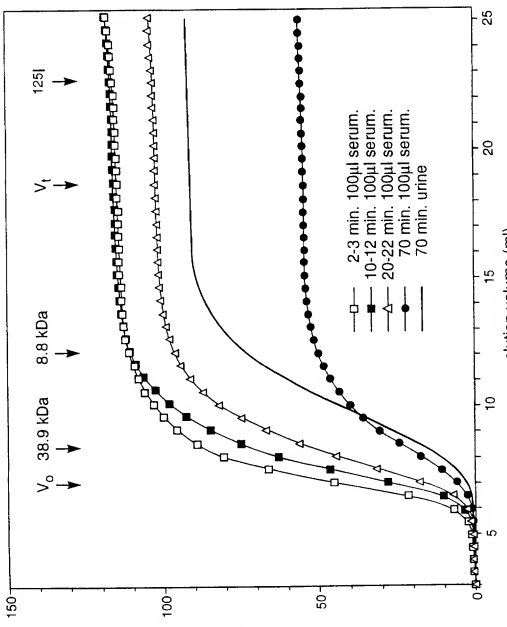

cumulative radioactivity (kcpm)
150
100
50
0
5
10
15
20
25
elution volume (ml)
V_o
38.9 kDa
8.8 kDa
V_t
125I
2-3 min. 100µl serum.
10-12 min. 100µl serum.
20-22 min. 100µl serum.
70 min. 100µl serum.
70 min. urine

FIG. 9

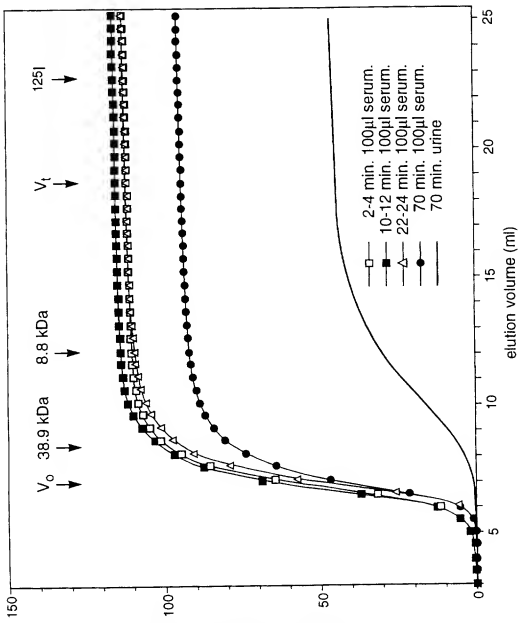

V_o
38.9 kDa
8.8 kDa
V_t
125I
2-4 min. 100µl serum.
10-12 min. 100µl serum.
22-24 min. 100µl serum.
70 min. 100µl serum.
70 min. urine
cumulative radioactivity (kcpm)
elution volume (ml)
0
50
100
150
5
10
15
20
25

FIG. 10

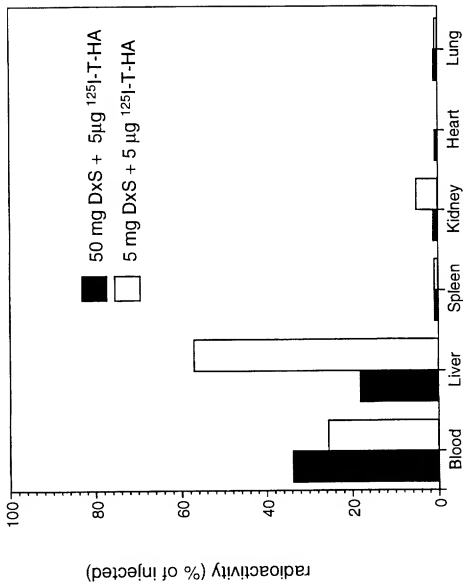

50 mg DxS + 5µg 125I-T-HA
5 mg DxS + 5 µg 125I-T-HA
radioactivity (% of injected)
100
80
60
40
20
0
Blood
Liver
Spleen
Kidney
Heart
Lung

FIG. 11

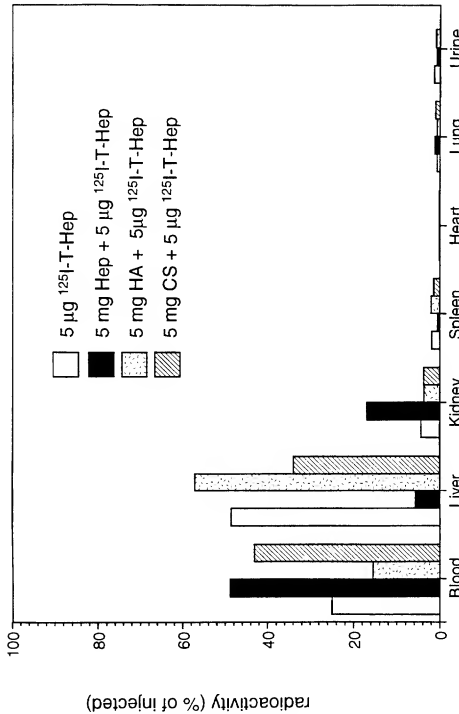

radioactivity (% of injected)
100
80
60
40
20
0
Blood
Liver
Kidney
Spleen
Heart
Lung
Urine
5 µg 125I-T-Hep
5 mg Hep + 5 µg 125I-T-Hep
5 mg HA + 5µg 125I-T-Hep
5 mg CS + 5 µg 125I-T-Hep

FIG. 12

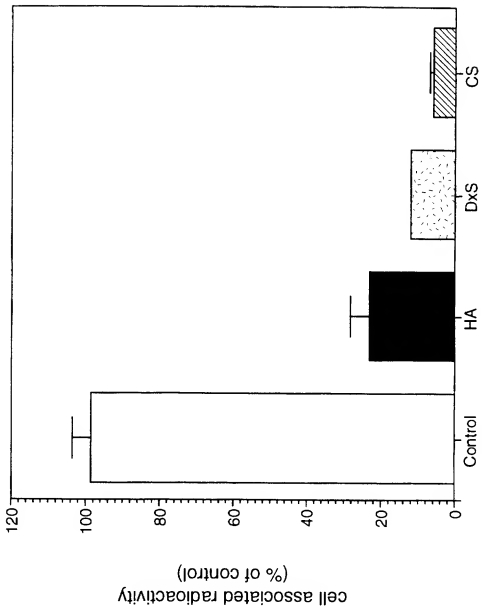

cell associated radioactivity
(% of control)
120
100
80
60
40
20
0
Control
HA
DxS
CS

FIG. 13

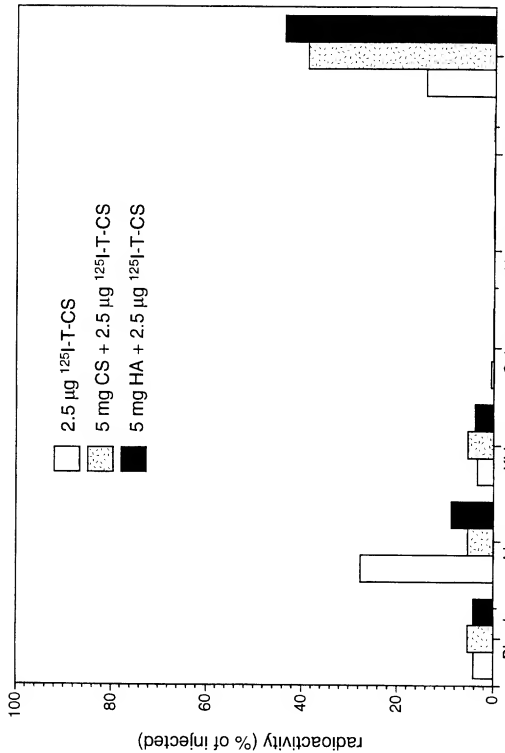

radioactivity (% of injected)
100
80
60
40
20
0
Blood
Liver
Kidney
Spleen
Heart
Lung
Urine
2.5 µg 125I-T-CS
5 mg CS + 2.5 µg 125I-T-CS
5 mg HA + 2.5 µg 125I-T-CS

FIG. 14

INTERNATIONAL SEARCH REPORT

Inte..ational Application No
PCT/CA 96/00793

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K31/725 //(A61K31/725,A61K31:715,A61K31:00)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 91 04058 A (NORPHARMCO INC) 4 April 1991 cited in the application see the whole document ---	1-45
A	CLINICAL CANCER RESEARCH, 1996, 9, 1607-1618, PHILADELPHIA, USA, XP000651805 ASSMANN V ET AL: "DIFFERENTIAL EXPRESSION OF THE HYALURONAN RECEPTOR CD44 AND RHAMM IN HUMAN PANCREATIC CANCER CELLS" see the whole document ---	1-45
P,A	WO 96 06622 A (HYAL PHARMA CORP ;ASCULAI SAMUEL SIMON (CA)) 7 March 1996 cited in the application see the whole document -----	1-45

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
17 March 1997	04. 04. 97
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+ 31-70) 340-3016	Authorized officer Herrera, S

Form PCT ISA 210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/CA 96/00793

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9104058 A	04-04-91	AP 175 A	03-04-92
		AT 131068 T	15-12-95
		AU 674894 B	16-01-97
		AU 5227493 A	03-03-94
		AU 6433090 A	18-04-91
		CA 2042034 A	22-03-91
		CN 1051503 A	22-05-91
		DE 69024039 D	18-01-96
		DE 69024039 T	13-06-96
		EP 0445255 A	11-09-91
		EP 0656213 A	07-06-95
		ES 2080837 T	16-02-96
		HU 64699 A	28-02-94
		HU 9500656 A	28-11-95
		JP 4504579 T	13-08-92
		LT 1582 A,B	26-06-95
WO 9606622 A	07-03-96	CA 2145605 A	28-09-96
		AU 3159595 A	22-03-96
		CN 1130532 A	11-09-96
		ZA 9507223 A	01-04-96
		WO 9407505 A	14-04-94
		WO 9526193 A	05-10-95
		WO 9529683 A	09-11-95
		WO 9530423 A	16-11-95